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ORIGINAL RESEARCH ARTICLE

Molecular study on the dengue virus in *Aedes aegypti* from Al-Madinah, Saudi Arabia using one step real time RT-PCR

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Abstract. *Aedes aegypti* mosquitoes are primary indigenous vector of recent urban dengue fever virus (DENV) outbreaks in Jeddah and Makkah, Saudi Arabia a neighborhoods' cities to Al-Madinah Al-Munawwarah. Virus surveillance in field caught *Aedes* mosquitoes using real time RT-PCR is a rapid, highly sensitive, efficient method to early predict the risk of dengue infection in a given area. To report the DENV activity reported in Al-Madinah for the first time. A total of 463 *A. aegypti* mosquitoes were collected (300 females) from Al-Madinah Al-Munawwarah area through an entomological survey for a year from July 2008 to June 2009, where their prevalence and seasonal distribution were reported. Female mosquitoes were pooled (up to 10 mosquitoes/pool) according to date and site of collection, and were kept at -80°C till screened for the presence of DENV RNA. They were homogenized using metal beads; viral RNA was extracted from pooled samples homogenate, and then assayed using the DENV general-type real time reverse transcriptase-polymerase chain reaction (RT-PCR) kit (ZJ Bio-Tech, Shanghai, China) in a single step procedure. No DENV was detectable in any of the tested female pools. The obtained results ruled out the possibility of introduction of DENV by *A. aegypti* to the area of Al-Madinah Al-Munawwarah.

Keywords: *Ae. Aegypti;* Al-Madinah Al-Munawwarah; DENV RNA; Real time RT-PCR.

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Introduction

A. aegypti has a widespread occurrence throughout the world at tropics and subtropics (Gubler, 1998). Being an urban domestic mosquito, it exists inside and near human habitation and prefers densely populated areas (Christophers, 1960;

Clements, 1999). *A. aegypti* is one of the most efficient mosquito vectors for arboviruses because it is highly anthropophilic and endophilic. *A. aegypti* mosquitoes are the most important and the primary domestic vector of urban dengue fever virus (Gubler, 1998). Dengue is an acute febrile viral disease caused by different serotypes (DEN1, DEN2, DEN3 and DEN4) which associated with headaches, bone, joint and muscular pains, rash and leucopenia (WHO, 1998). Dengue is transmitted by adult *A. aegypti* females, which require blood to complete oogenesis. The female mosquito ingests viremic blood containing the dengue viruses from infected humans; then the virus replicates in the mosquito and establishes a persistent infection in the mosquito salivary glands to be re-injected again in the blood of susceptible humans (Christophers, 1960; Clements, 1999).

A. aegypti was established as the primary indigenous vector of recent urban DENV outbreaks in Jeddah and Makkah, Saudi Arabia (Ghaznawi et al., 1997; Fakeeh and Zaki, 2003; Ayyub et al., 2006; Khan et al., 2008) nearby cities to Al-Madinah. The DENV activity has never been reported in Al-Madinah.

Identification and serotyping of DENV in infected mosquitoes is made by virus isolation through the gold standard cell culture-based test followed by serotyping (Khan et al., 2008). However, the method has limitations; it takes a long time (about a week), requires a well-equipped laboratory and has low sensitivity. It was replaced by molecular diagnosis based on reverse transcription (RT)-PCR using conventional PCR and real time RT-PCR (Kuno, 1998; Harris et al., 1998; Guzman and Kouri, 2004).

Real-time RT-PCR has gradually replaced the virus isolation method as the standard for the detection of DENV. It has many advantages compared to the conventional PCR, including rapidity, ability to provide quantitative measurements, lower contamination rate, higher sensitivity, higher specificity and easy standardization.

Previously, we failed to detect DENV in 55 female mosquitoes collected from Al-Madinah from May to June 2008 using real time RT-PCR assay. We related this to the small number of collected and examined mosquitoes. The present work had the objective to assess the possibility of DENV transmission by field caught *A. aegypti* mosquitoes in Al-Madinah depending on relative large samples. An entomological survey was conducted for a year through which abundance of *Aedes* mosquitoes, diversity of their populations and seasonal variation were documented (El-Badry and Al-Ali, 2010) and the resulting female populations were examined, using single step real time RT-PCR technique.

Materials and methods

Study area and mosquitoe collection and identification

Al-Madinah Al-Munawwarah, Western region of Saudi Arabia is located approximately 420 Km North of Jeddah and Makkah neighborhoods' cities with continuity of dengue sporadic cases (Fakeeh and Zaki, 2003; Ayyub et al., 2006; Khan et al., 2008). This led us to conduct an entomological survey in Al-Madinah, where prevalence and seasonal distribution of *A. aegypti* mosquitoes were reported (under publication). Adult *Aedes* were collected for a year from July 2008 overnight using standardized black hole UV trap that produce CO_2 through the spray-sheet method. A total of 463 *A. aegypti* mosquitoes were collected (300 females and 163 males) from the basement of buildings under constructions surrounded by inhabited buildings and farms in Al-Madinah Al-Munawwarah area. Captured insects were kept in a humid and cool environment and rapidly transported to the laboratory where they were immediately segregated and sorted by sex. Male *Aedes* were stored in 1.5 ml vials containing 95% ethanol, latter were mounted in Hoyer's media for taxonomic identification. Females were examined for feeding status, and parity then pooled (up to 10 mosquitoes/pool) according to the date and the site of collection and stored at -80°C until processed and examined for the presence of DENV.

Molecular detection of DENV

- *RNA extraction*

DENV was extracted from pooled samples by using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany; code no. 5052904) according to the manufacturer`s instructions. The method based on the use of QIAamp spin column and ethanol precipitation. In brief, a pool up to 10 collected mosquitoes were gathered and grinded using metal beads, mixed with 200 µl of working solution and 50 µl of proteinase K before incubation for 10 minutes. Then, 100 µl isopropanol were added and the entire mixture was transferred to a spin column and centrifuged at 10000 rpm for 1 minute. The bound RNA was washed twice with wash buffer. RNA was then eluted from the column by the addition of 50 µl of elution buffer and centrifuged at 10000 rpm for 1 minute. The RNA was finally eluted in a final volume of 60µl of buffer AVE and stored at -70°C till further use. The purity of RNA was assessed by UV spectrometry; an A_{260}/A_{280} ratio of 1.7-2.2 was typically obtained.

- *Probe and primers sequences for DENV used in the study*

The sequence and length of the forward and reverse primers and probe used in this study are shown in table 1.

Table 1. Probe and primer sequences for DEN virus group-specific real-time PCR assay

	Sequence (5`-3`)	Size (bases)	Nucleotide position
Forward primer	5'-GATAGACCAGAGATCCTGCTGTCT-3'	19	10635–10658
Reverse primer	5'-ACCATTCCATTTTCTGGCGTT-3'	14	10708-10682
TaqMan MGB Probe	5'-AGCATCATTCCAGGCAC-3'	18	10663–10679

- *Real time RT-PCR assay*

The assay was done using the DENV general-type real time reverse transcriptase PCR kit (ZJ Bio-Tech, Shanghai, China) in single step procedure according to the manufacturer`s instruction on the StepOne™ real-time PCR machine (Applied Biosystems 7700, USA). During the PCR reaction, DNA polymerase cleaves the DNA probe at the 5`end and separates the reporter dye (FAM) from the quencher dye (NFQ-MGB) only when the DNA probe hybridizes to the target DNA. This result in fluorescent signal generated by cleaved reporter dye, which monitored on real-time by the PCR detection system. The PCR cycle at which an increase in the fluorescence signal is detected initially (CT) is proportional to the amount of the specific PCR product. A 5 µl of consensus positive control (supplied within the kit) was used to monitor the success of amplification and it is matched with the DENV different serotypes.

The reaction was done in one step real time RT-PCR. This reaction involved combined reverse transcription (RT) and DNA amplification processes. The Applied Biosystems StepOne(™) real-time PCR machine was used, Foster City, San Diego, USA. Possible PCR inhibition was identified by measuring the VIC/JOE fluorescence of the internal control (IC). An external positive control (standard 1×10^7 copies/ml) contained, allowed the quantification of the gene load. All data were investigated through the use of step one plus software. The RT-real time PCR was performed using the following program according to the manufacturer as the followings: 45°C for 10 min. for 1 cycle, followed by 95°C for 15 min. for 1 cycle, then 95°C for 15 sec., 60°C for 60 sec. for 40 cycles. The fluorescence was measured at 60°C.

Results

A total of 463 *A. aegypti* adult mosquitoes were identified; 300 (64.8%) females and 163 (35.2%) males (figure 1). Figure 2 shows the standard calibration curve of DENV RNA using serial dilutions. No DENV was detected in any of the mosquito pools tested by real time RT-PCR assay (figure 2).

The results were negative as there was undetectable signal in the FAM channel, at the same time, a VIC/JOE signal from the IC was noticed. This mean that, the samples didn`t contain any DENV RNA. The detected signal of the internal control ruled out the possibility of PCR inhibition.

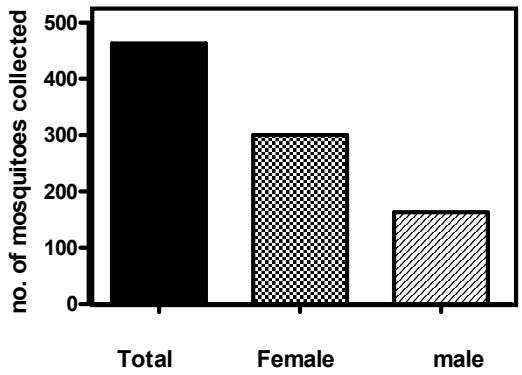

Figure 1. The numbers of *Aedes aegypti* mosquitoes collected from July 2008 to June 2009, showed the collection of 300 (64.8%) females and 163 (35.2%) males. The total number was 463.

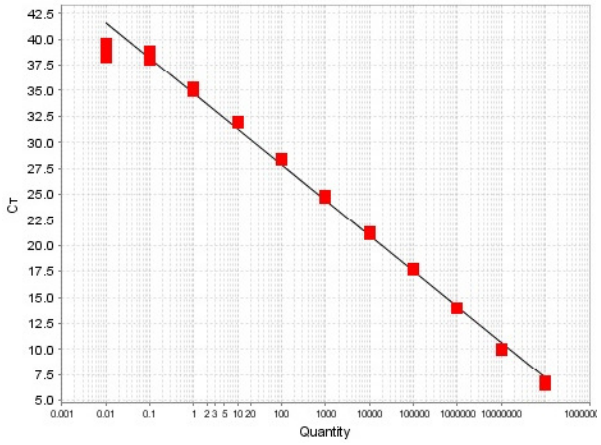

Figure 2. The real time RT PCR standard calibration curve of DENV RNA general type using serial dilutions (1 x 10^7 copies/ml).

Discussion

Aedes aegypti was established as the primary indigenous vector of recent urban DENV outbreaks with continuity of dengue sporadic cases in Jeddah and Makkah, Western Saudi Arabia (Fakeeh and Zaki, 2003; Ayyub et al., 2006; Khan et al., 2008), nearby cities to Al-Madinah Al-Munawwarah. However DENV activity has never been reported in Al-Madinah. Many field studies (Rosen et al., 1983; Rosen et al., 1985; Thenmozil et al., 2000; Kow et al., 2001; Diallo et al., 2003) and laboratory studies (Joshi et al., 2002; Vazeille et al., 2003) documented the occurrence of transovarial and venereal transmission of DENV by *A. aegypti* (Rosen, 1987) which are both important factors in maintaining the DENV in vector and introduce the DENV to a new community.

We reported the presence of *A. aegypti* as the only identified species in Al-Madinah through an entomological survey for a year from July 2008, where their prevalence and seasonal variations were documented (El-Badry and Al-Ali, 2010). Assuming that *A. aegypti* was introduced to Al-Madinah from neighborhoods cities (Jeddah and Makkah) with possibility of being infected by DENV.

To rule out the possibility of introduction of DENV and its maintenance in Al-Madinah community by field caught *A. aegypti* through vertical and/or venereal transmission. Field captured females (300) were examined for possibility of their infection by DENV infected females the single step reverse transcriptase real time PCR assay as a molecular reliable method.

The most common method for dengue virus (DENV) quantitation is the plaque assay, but there are DENV strains that do not form plaques. Due this reason, a PCR protocol able to detect and quantify DENV in the different kinds of samples was employed. Several real-time PCR based methods for detection of DENV have been reported in the last decade. These assays have targeted the 3'UTR (Houng et al., 2001; Chien et al., 2006), NS5 (Laue et al., 1999; Callahan et al., 2001), core (Chien et al., 2006) and the envelope (Ito et al., 2004) gene sequences. The majority of the recent reports describe development of the serotype specific real time PCR for dengue using TaqMan probes (Johnson et al., 2005; Sadon et al., 2008) or FRET probes (Lai et al., 2007).

Serotype-specific real time RT-PCR tests are appropriate, rapid and highly sensitive compared to nested RT-PCR which is time consuming as it needs post processing and

amplification and it might elaborated false positive results due to contamination and prone to misinterpretation due to occurrence of subjecting to electrophoresis. Also false negative results due to PCR failure due to mismatches in primers and DNA probes with nested PCR can be avoided in the real time RT-PCR by including highly conserved regions of DENV genome for designing primers and probes (Poersch et al., 2005). In addition, due to low virus infection among mosquitoes, virus surveillance require pooling of large mosquito populations, real time RT-PCR assay is sufficiently sensitive and specific enough to detect just a single DENV infected mosquito from a large pool, and could be established for rapid detection of DENV within their vector mosquitoes (Perera et al., 2009).

Virus surveillance in field caught *Aedes* mosquitoes using real time RT-PCR is a rapid, highly sensitive, efficient method to early predict the risk of dengue infection in a given area as infected *A. aegypti* can be detected as early as six weeks before the start of DENV outbreaks (Rosen and Gubler, 1974; Lifson, 1996; Chow et al., 1998; Gubler, 1998). Furthermore, this assay is simple and does not require gel documentation equipments, so it could be used as a field method for surveillance of DENV-infected mosquitoes (Perera et al., 2009). The obtained results ruled out the possibility of occurrence of the DENV by *A. aegypti* to the area of Al-Madinah Al-Munawwarah and suggest that transovarial transmission of DENV in mosquitoes occurs at a very low (if any) frequency and may have no role in the maintenance of DENV in *A. aegypti* population in Al-Madinah, Saudi Arabia. According to the records of Saudi Ministry of Health, there are a correlation between pilgrimage and introduction of dengue fever. The dengue fever is prevalent in Mecca and Jeddah; however, there is no evidence (as reported in our study) in Al-Madinah. This may be attributed to the fact that distance between Jeddah and Mecca is around 70 km, but the distance between Al-Madinah and Mecca is 420 Km. This long distance may have a role in reducing the possibility of transmission of dengue outbreaks in Al-Madianh. Besides, the strict regulation by the local authorities in Al Madianh may attenuate the dengue transmission. Therefore it is unlikely to be a decisive factor in dengue establishment and persistence in Al-Madinah urban environment. However the possibility of introduction of DENV by this competent vector necessitates further epidemiological surveillance, and implicates regular strict monitoring of *Ae. aegypti* in Al-Madinah.

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